BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF SOME *TRICHODERMA* ISOLATES ANTAGONISTIC TO *RHIZOCTONIA SOLANI* THE CAUSAL OF BEAN ROOT-ROT

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ABSTRACT:

The genus *Trichoderma* is used as a bioagent against many of soil-borne plant pathogenic fungi. Several potential isolates for biological control are difficult to be distinguished from the others. In this investigation, proteins and randomly amplified polymorphic DNA (RAPD) markers were used to estimate the genetic variations between six isolates of *Trichoderma* spp which were. previously isolated from the rhizospheres of different plants growing in Fayoum Governorate, and two reference strains (*T. harzianum* and *T. koningii*) obtained from Faculty of Agriculture, Ain Shams University. Four *Trichoderma* isolates were characterized morphologically as *T. harzianum*, one as *T. koningii* and one as *T. viride*. The best antagonistic effect against *Rhizoctonia solani* was obtained from some *T. harzianum* isolates. The damping off disease of bean seedlings was effectively controlled by adding either *T. harzianum* or their culture filtrates to the soil infested with *R. solani* under greenhouse conditions. The results of biochemical and molecular analysis revealed 96.8% polymorphism for proteins and 87% for RAPD. The similarity indices ranged from 77.8 % to 29.6% and 69% to 32% for proteins and RAPD, respectively. Cluster analysis based on similarity matrices of protein markers separated *Trichoderma viride* (FE_6) from all the other isolates While, the other seven isolates fall in a second cluster. Cluster analysis of RAPD markers separated all *T. harzianum* isolates, FE_1, FE_2, FE_3, FE_4 and the reference strain (I_8) in one cluster, while the other three isolates fall in a second cluster. The protein markers were successful in identifying 4 out of the eight *Trichoderma* isolates with 5 isolate specific unique markers while, RAPD assay (using 9 random primers) identified 8 out of the eight isolates with 37 isolate specific unique markers. From the obtained results, it is concluded that the RAPD-PCR analyses could be successfully used to characterize and determine specific molecular markers for the *Trichoderma* isolates.

Key words: *Trichoderma* fungus, morphological characters, biocontrol, protein markers and RAPD markers.

INTRODUCTION

The genus *Trichoderma* (Ascomycetes, Hypocreales) is a filamentous fungus widely distributed in the soil, plant material, decaying vegetations and wood. Species in this genus are of great economic importance as sources of enzymes, antibiotics, plant growth promoters, xenobiotic degraders and as most commercial biofungicides (**Latha, *et al*., 2002; Marco *et al*., 2004; Ozbay and Newman, 2004 and Thornton, 2005).**

Rhizoctonia solani is a common soil-borne pathogen infecting several crops allover the world including Egypt. There are many different methods for controlling this pathogen; *i. e.* crop rotation, resistant varieties and treatment of seeds and/or soil with fungicides. The controlling of *R. solani* become unsuitable or not effective, mainly due to its genetic variability, high efficacy to survive in

the soil and seeds for long periods and due to its physiological flexibility to having a wide host-range **(Leach and Garber, 1970).**

The use of antagonistic microorganisms against *R. solani* had been investigated as an effective alternative control method. The capability of *Trichoderma* spp. to control *R. solani* considerably varies and it is possible to improve its biological control efficiency by the selection of isolates with high antagonistic potential and adapted to certain ecological or geographical areas **(Papavizas, 1985).**

The exact characterization and identification of *Trichoderama* isolates to the level of species is the first step in utilizing the full potential of fungi in a specific application. The morphological characters of *Trichoderama* had been discussed by **Rifai (1969) and Bissett (1991).** They emphasized the difficulties inherent in defining morphological species of *Trichoderama.* **Samuels (1996)** also provided detailed observations and comments on the utility of morphological characters to define species in *Trichoderama*.

The molecular analysis of several strains revealed that the classification based on morphological data had been to a great extent, erroneous resulting in re-classification of several isolates and species **(Meyer *et al*., 1993; Rehner and Samuels, 19995; Kuhls, *et al*., 1996).** The physiological and phenotypic characters, isozyme and molecular markers were used to identify *Trichoderama* spp. **(Druzhinina and Kubicek, 2005).**

The present investigation aimed to study the genetic variability of *Trichoderama* isolates using their antagonistic potential against *Rhizoctonia solani*. The protein fingerprinting and RAPD technique, as well as the relationship between their antagonistic capability and RAPD profiles were analyzed.

MATERIALS AND METHODS:

Fungal Isolates:

Six isolates of *Trichoderma* spp. Nos. FE_{1-6} were isolated from the rhizospheres of different plants growing in Fayoum Governorate and two reference strains (*T. harzianum* and *T. koningii*) were obtained from (MERCEN), Faculty of Agriculture, Ain Shams University (Table 1). The soil-borne pathogen, *Rhizoctonia solani* was isolated from the roots of bean plants showing typical root rot symptoms.

Table (1): Isolates of *Trichoderma* used in the present investigation.

Isolate No.	Host	Origin
FE_1	Cucumber	Fayoum Governorate, Egypt
FE_2	Faba bean	Fayoum Governorate, Egypt
FE_3	Bean	Fayoum Governorate, Egypt
FE_4	Bean	Fayoum Governorate, Egypt
FE_5	Cowpea	Fayoum Governorate, Egypt
FE_6	Cucumber	Fayoum Governorate, Egypt
I_7	-	MERCEN, Fac. Agric., Ain Shams Univ.
I_8	-	MERCEN, Fac. Agric., Ain Shams Univ.

Isolation and Identification of the Tested Fungal Isolates:

To study the microbial flora in rhizosphere of bean, cowpea, cucumber and faba bean plants, the method developed by **Louw and Webely (1959)** was followed. Pure cultures of *Trichoderma* and *R. solani* isolates were obtained using single spore or hyphal tip techniques described by **Brown (1924) and Riker and Riker (1936).** The obtained isolates were identified according to

Rifai (1969) and Bissett (1984 and 1991). After growing on PDA at 25 °C for four days, the isolates were microscopically carefully inspected.

Antagonistic Capability of *Trichoderma* Isolates:

The antagonistic effect of the *Trichoderma* isolates against *R. solani* was investigated according to **Dennis and Webster (1971)** under both laboratory and greenhouse conditions**.** The isolates were grown in Petri-dishes containing PDA medium for seven days. Discs (5 mm in diameter) were cut from the edge of the fungal growth and transferred to another Petri-dish with PDA. Each plate received two discs (one from *Trichoderma* and the other from *R. solani*) at the same time or at different times according to the growth of *Trichoderma* isolates. The discs were placed at a distance of 7 cm from each other. The plates were incubated at 26 °C. The resulted inhibition percentage was calculated using the following equation:

Inhibition Percentage (IP) = {(C –T) /C } x 100.

where (C) is the mean diameter (mm) of the growth in the control treatment and (T) is the mean diameter (mm) of the growth in the treatment tested.

Soil infestation was carried out either with *Trichoderma* isolates grown on sorghum, sand, water (SSW) medium or by adding their culture filtrate under greenhouse conditions to study their effects on the incidence of bean root rot disease caused by *R. solani*. Soil was infested by the inoculum of *R. solani* (5%) and each *Trichoderma* isolates grown on SSW medium. Also, the *Trichoderma* culture filtrates (20 ml/pot) were added to the soil infested by *R. solani* at the second leaf stage. Data were expressed as percentage of damping off and survival plants **(Riker and Riker, 1936).** The pots (12 cm diameter) were sown by bean seeds (5/pot) and irrigated as usual.

The antagonistic experiments were designed as complete randomized block design with ten replications. The obtained data were statistically analyzed **(Snedecor and Cochran 1980),** differences between means were tested using Least Significant Differences (LSD) method at 5% level. The data obtained from greenhouse experiment were transformed according to (**Steel and Torri, 1960**)

Protein Banding Patterns:

Erlenmeyer flasks 250 ml containing 100 ml Potato Dextrose Broth (PDB) medium were inoculated by discs (7 mm in diameter) taken from 48 hours-old culture of any of *Trichoderma* isolates tested. Flasks were shaken on a rotary shaker (120 rpm) for 7 days at room temperature (27 °C) in the dark. The liquid content of each flask was passed through a filter paper, Whatman No.1 using a vacuum pump in order to obtain the fungal biomass. The obtained fungal growth for each isolate was frozen in liquid nitrogen, freeze-dried for 8 hr under vacuum and kept at -80 °C before protein extraction. The soluble mycelial proteins were extracted in phosphate buffer pH 7.0 {1 sample: 4 buffer (w/v)}. The homogenate was transferred into 4 ml plastic tubes and centrifuged at 2000 rpm for 45 min at 4 °C using HERMLE Z323K centrifuge. The supernatant was kept at -20 °C until use. Total soluble protein concentration in the fungal extracts was determined according to **Lowery *et al*. (1951).**

Fractionation of soluble mycelial proteins was carried out in one dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to **Laemmli (1970)**. The fungal protein extract was mixed with 5X sample buffer in microcentrifuge tubes and boiled in a water bath for 5 min. The denatured proteins were cooled at room temperature, then fifty micrograms protein were loaded into each well in stacking gel (2.5% w/v). The resolving gel was 10% (w/v). The protein fractionation was performed at a constant voltage of

100 V, 4 °C for 4 hr. The gel was stained in silver staining solutions and the development was stopped by rinsing in water three times **(Giulian *et al*., 1983)**.

Randomly Amplified Polymorphic DNA (RAPD):

The extraction of total genomic DNA of each *Trichoderma* isolate was done according to **El-Fiky (2003).** The primers OPA, as well as Taq DNA polymerase and the dNTPs were supplied by Operon Technologies. Nine random, 10-mer primers (Table, 2) were used in the detection of polymorphism among the eight *Trichoderma* isolates. PCR reactions were conducted according to **Williams *et al*. (1990).** Amplification reactions were carried out in a total volume of 25 µl containing 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM primers, 0.2U/µl Taq DNA polymerase and 100 ng template DNA. The amplification process was accomplished in a thermocycler UNO II (Biometra) programmed to fulfill 40 cycles after an initial denaturation cycle for 4 min at 94°C. Each cycle consisted of a denaturation step at 94 °C for 1 min, an annealing step at 36 °C for 1 min, and an elongation step at 72 °C for 2 min. The primer extension segment was extended to 7 min at 72 °C in the final cycle, then 4 °C. The amplification products (7 µl) were mixed with 3 µl loading buffer and separated on 1.5 % agarose gel containing ethidium bromide (0.5 µg/ml) in 1 X TAE buffer at 100 volts for 1.5 hr. PCR products were visualized on ultraviolet light and photographed using a Polaroid camera. The DNA fragment sizes were determined by comparisons with the 1kb DNA ladder marker.

Table (2): Sequence of the nine decamer arbitrary primers used in RAPD-PCR.

No.	Primer	Sequence 5`…3`
1	A02	TGCCGAGCTG
2	A03	AGTCAGCCAC
3	A04	AATCGGGCTG
4	A06	GGTCCCTGAC
5	A07	GAAACGGGTG
6	A16	AGCCAGCGAA
7	A18	AGGTGACCGT
8	A19	CAAACGTCGG
9	A20	GTTGCGATCC

Data Analysis:

The banding patterns generated by protein and RAPD-PCR markers analysis were compared to determine the genetic relatedness of the eight *Trichoderma* isolates. The genetic similarity coefficient between each two isolates was estimated according to Dice coefficient **(Nei and Li, 1972).** The similarity matrix was used in the cluster analysis using RAPDistance software package, version 1.04.

RESULTS AND DISCUSSION

I- Morphological Characterization of *Trichoderma* Isolates:

The six isolates of *Trichoderma* were characterized by using a distinctive morphology including rapid growth, bright green or white conidia, pigments, repetitively branched and conidiophore. Data presented in Table (3) show that the most common group comprising 66.6% of the isolates was characterized as *Trichoderma harzianum* strain. This group included isolates Nos. FE_1, FE_2, FE_3, and FE_4, while the isolates Nos. FE_5 and FE_6 were characterized as *T. koningii* and *T. viride* strains, respectively.

Table (3): The morphological characterization of *Trichoderma* isolates, isolated from Fayoum Governorate.

Isolate No.	Host	Characterization
FE_1	Cucumber	*T. harzianum*
FE_2	Faba bean	*T. harzianum*
FE_3	Bean	*T. harzianum*
FE_4	Bean	*T. harzianum*
FE_5	Cowpea	*T. koningii*
FE_6	Cucumber	*T. viride*

II- Antagonistic Capability of *Trichoderma* Isolates Against *Rhizoctonia solani*

A-*In vitro*

The previously characterized *Trichoderma* isolates were investigated *in vitro* to determine their antagonistic effect against *R. solani* (Table, 4 and Fig. 1A). Data presented in Table (4) show that the best antagonistic effect against *R. solani* was obtained from *Trichoderma* isolates, FE_1 and FE_3. The corresponding inhibition percentages were 46.11 and 43.89, respectively when adding the discs of *Trichoderma* isolates and *R. solani* at the same time. Whereas, *it was* 26.56 and 23.56 inhibition percentage resulted in *Trichoderma* isolates, FE_5 and FE_2, respectively when the two microorganisms were added in different times.

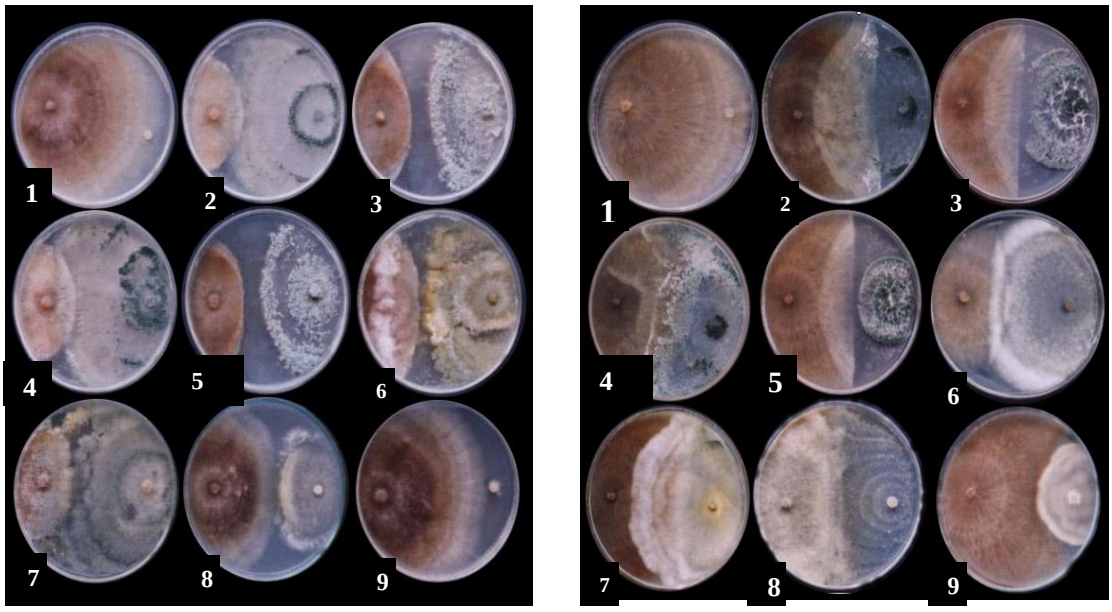
Results presented in Fig. (1A) clearly show that *Trichoderma* isolates, FE_1 and FE_3 resulted in the highest inhibitory effect against *R. solani*. No inhibition zone was observed between the cultures of *Trichoderma* and *R. solani*. As shown in Fig. 1B, the *Trichoderma* isolates, FE_5 and FE_2 clearly showed an antagonistic behaviour against *R. solani*, whereas *Trichoderma* isolates, FE_1 and FE_3 showed a weak antagonistic effect towards growth of *R solani*.

The data showed that the best antagonistic effect against the pathogen was obtained from *T. harzianum* isolates. These results are in agreement with **Elad *et al.*, (1980) and Mathew and Gupta, (1998)**. They found that the mycelial growth of *R. solani* was strongly inhibited *in vitro* by the antagonist *T. harzianum*.

Table (4): The antagonistic effect of *Trichoderma* isolates on the mycelial growth (MG) of *Rhizoctonia solani*.

***Trichoderma* isolate**	**Cultivation at the same time**		**Cultivation at different times**	
	MG (mm)	**IP.**	**MG (mm)**	**IP.**
R. solani	90.0	0.0	90.0	0.0
FE_1+ *R. solani*	48.5	46.11	76.5	15.00
FE_2+ *R. solani*	56.0	37.78	68.8	23.56
FE_3+ *R. solani*	50.5	43.89	75.8	15.78
FE_4+ *R. solani*	55.0	38.89	70.9	21.22
FE_5+ *R. solani*	67.5	25.00	66.1	26.56
FE_6+ *R. solani*	56.0	37.78	75.9	15.67
I_7+ *R. solani*	68.5	23.89	70.4	21.78
I_8+ *R. solani*	72.5	19.45	72.7	19.23
L.S.D. at 0.05	**0.09**		**1.60**	

IP: the inhibition percentage



(A) (B)

Fig. (1): Antagonistic effect of eight *Trichoderma* isolates against *Rhizoctonia solani in vitro*, cultivation at the same time (A) and at different times (B).

1- *R. solani*
2- *Trichoderma* isolat, FE_1 + *R. solani*
3- *Trichoderma* isolat, FE_2 + *R. solani*
4- *Trichoderma* isolat, FE_3 + *R. solani*
5- *Trichoderma* isolat, FE_4 + *R. solani*
6- *Trichoderma* isolat, FE_5 + *R. solani*
7- *Trichoderma* isolat, FE_6 + *R. solani*
8- *Trichoderma* isolat, I_7 + *R. solani*
9- *Trichoderma* isolat, I_8 + *R. solani*

B- Under greenhouse conditions

The antagonistic capability of *Trichoderma* isolates against *Rhizoctonia solani* was also investigated under greenhouse conditions using two methods of soil inoculations; *i.e.* soil infestation with the inoculum of *Trichoderma* isolates grown on sorghum, sand, water (SSW) medium and culture filtrate of *Trichoderma* isolates grown on PDB medium. Data illustrated in Table (5) and Fig. (2) proved the antagonistic effect of the desired *Trichoderma* isolates under greenhouse conditions using the two methods for inoculations. Concerning *Trichoderma* isolates grown on SSW, the highest reduction in percentage of damping off was obtained from FE_1 and FE_3. Regarding the second method, *Trichoderma* isolate, EF_1 resulted in the same trend in minimizing the damping off of the tested disease. Using culture filtrate of *Trichoderma* isolates, FE_1 and FE_3 resulted in the lowest percentages of damping off being 28% and 32%, respectively. The percentages of the survival plants with culture filtrates of FE_1 and FE_3 isolates were 72 % and 68 %, respectively.

Table (5): Effect of treating soil either with 8 different isolates of *Trichoderma* or their culture filtrates on the incidence of bean root rot disease caused by *R. solani* (greenhouse conditions).

Treatment	Inoculum grown on SSW				Fungal culture filtrate			
	Damping off %		Survival Plants %		Damping off %		Survival Plants %	
	Untrans.	Trans.	Untrans.	Trans.	Untrans.	Trans.	Untrans.	Trans.
Control*	0	1.00	100	2.45	0.0	1.00	100	2.45
R. solani	86	1.75	14	1.27	52	1.89	48	1.83
FE_1 + *R.solani*	60	1.56	40	1.72	28	1.54	72	2.14
FE_2 + *R.solani*	76	1.67	24	1.47	40	1.72	60	1.99
FE_3 + *R.solani*	64	1.59	36	1.67	32	1.60	68	2.09
FE_4 + *R.solani*	72	1.66	28	1.54	38	1.69	62	2.02
FE_5 + *R.solani*	80	1.71	20	1.40	44	1.78	56	1.94
FE_6 + *R.solani*	68	1.63	32	1.60	34	1.60	66	2.07
I_7 + *R.solani*	82	1.73	18	1.35	46	1.81	54	1.92
I_8 + *R.solani*	84	1.74	16	1.32	48	1.84	52	1.89
LSD at 5%		**0.12**		**0.18**		**0.47**		**0.12**

The data showed that bean damping off disease caused by *R solani* was effectively controlled by adding either *Trichoderma* isolates or their culture filtrates to the soil infested with *R. solani* under greenhouse conditions. These present results are in agreement with **Hadar *et al.*, (1979)**, how showed that the damping off of bean, tomato and eggplant was effectively controlled by adding *Trichoderma* culture to the soil infested with *R. solani* under greenhouse conditions. In this respect, **Kucuk and Kvanc, (2003**) found that the filtrate of *T. harzianum* were effective against *Fusarium* sp., *R. solani*, *Sclerotium rolfsii*, and *Gaeumannomyces graminis*. **Ozbay and Newman, (2004**) pointed out that *Trichoderma* spp. are effective biological control agents of plant diseases caused by both soil-born and leaf-infecting plant pathogenic fungi. These *Trichoderma* were often very fast growing and rapidly colonize substrates.

Fig. (2): Antagonistic effect of *Rhizoctonia solani* in greenhouse by using soil infestation (A) or fungal filtrate of *Trichoderma* isolates (B).

1- Control
2- *R. solani*
3- *Trichoderma* isolat, FE_1 + *R. solani*
4- *Trichoderma* isolat, FE_2 + *R. solani*
5- *Trichoderma* isolat, FE_3+ *R. solani*
6- *Trichoderma* isolat, FE_4 + *R. solani*
7- *Trichoderma* isolat, FE_5 + *R. solani*
8- *Trichoderma* isolat, FE_6 + *R. solani*
9- *Trichoderma* isolat, I_7 + *R. solani*
10- *Trichoderma* isolat, I_8 + *R. solani*

III- Protein Banding Patterns

On using SDS–PAGE, the total proteins of the hyphal tissues were separated into 31 bands (one monomorphic and 30 polymorphic) according to their relative mobility (Rm) values in the eight *Trichoderma* isolates. The molecular weight of protein banding patterns ranged between 114.411 to 12.614 kDa (Figure 3). Generally, There were many clear differences between the different *Trichoderma* isolates. The total number of bands in each isolate were 19,16,17,18,22,8,13 and 16 for isolate No, FE_1, FE_2, FE_3, FE_4, FE_5, FE_6, I_7 and I_8, respectively. The eight *Trichoderma* isolates had one common band (band No. 22) with molecular weight of 30.187 kDa. On the other hand, band No. 14 with molecular weight 50.457 kDa. appeared in *Trichoderma* isolate EF_5 only and bands No. 26, 29 and 30 with molecular weights 24.009, 14.939 and 13.909 kDa disappeared in *Trichoderma* isolates, I_8, I_7 and FE_6, respectively. These four bands were used as isolate-specific markers.

The similarity coefficient percentage and the dendrogran of *Trichoderma* isolates based on Dice Coefficient and UPGMA analysis were shown in Table (6) and Figure (4). The strongest relationship scored between FE_2 and FE_4 isolates showed the similarity of 82.4%, while the lowest scored between FE_6 and FE_1 isolates showed the similarity of 29.6%.

The dendrogram was divided into two clusters. The first cluster, contained FE_6 isolate, while the second cluster contained the rest of *Trichoderma* isolates. The cluster was divided into two subclusters. The first subcluster includes I_7 isolate. The second subcluster was divided into two sub- subclusters. The first sub–subcluster includes I_8 isolate and the second sub–subcluster was divided into two groups. The first group was included FE_1 and FE_3 isolates. The second group was divided into two subgroups. The first subgroup included FE_5 isolate and the second subgroup included FE_2 and FE_4 isolates.

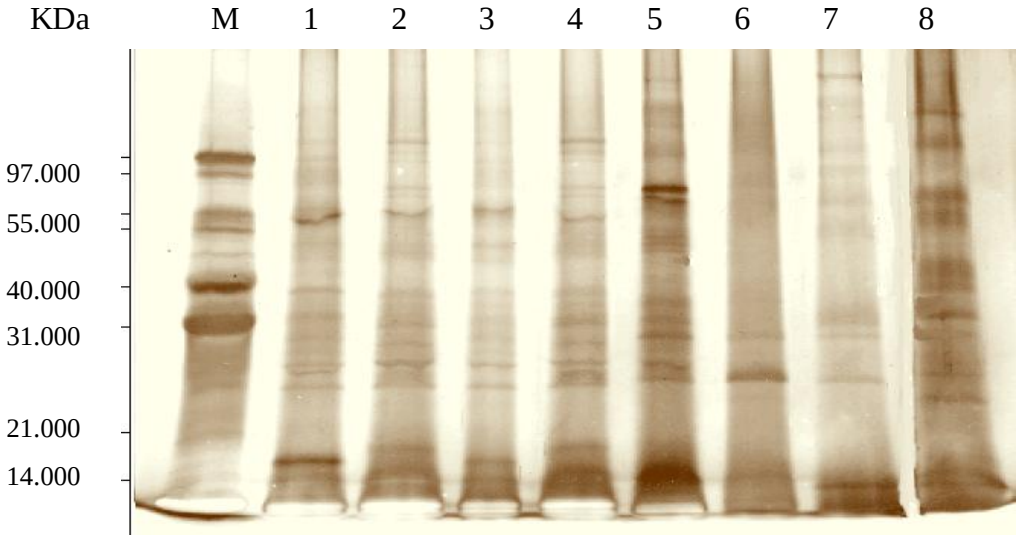


Fig. (3): Polyacrylamide gel of protein patterns in the eight *Trichoderma* isolates. Lanes 1-8 represent isolates FE_1, FE_2, FE_3, FE_4, FE_5, FE_6 I_7 and I_8, respectively. Lane M represents the molecular size marker.

Table (6): Similarity coefficient percentage among eight *Trichoderma* isolates based on SDS- PAGE.

Trichoderma Isolates	FE_1	FE_2	FE_3	FE_4	FE_5	FE_6	I_7	I_8
FE_1	**100.0**							
FE_2	68.6	**100.0**						
FE_3	77.8	66.7	**100.0**					
FE_4	70.3	82.4	57.1	**100.0**				
FE_5	58.5	68.4	71.8	70.0	**100.0**			
FE_6	29.6	41.7	32.0	38.5	33.3	**100.0**		
I_7	37.5	41.4	40.0	58.1	57.1	38.1	**100.0**	
I_8	68.6	62.5	60.6	58.8	57.9	41.7	41.4	**100.0**

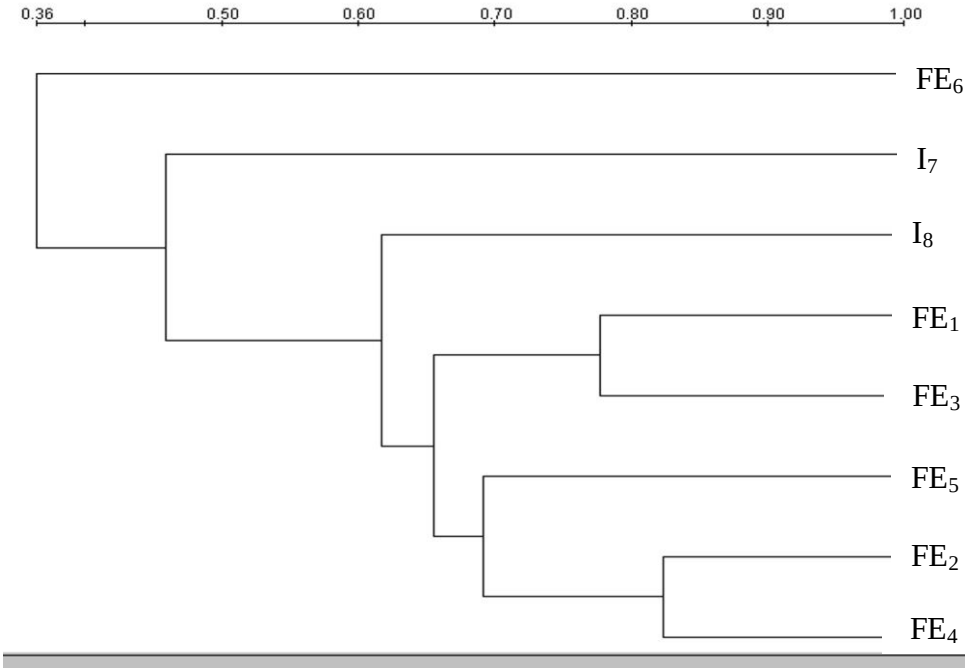


Fig. (4): Dendrogram among eight *Trichoderma* isolates based on SDS-PAGE.

Hyphal protein electrophoresis provides valuable evidence for taxonomic and evolutionary relationships of *Trichoderma* isolates (**Zhang *et al.*, 1993; Liu *et al.*, 1994; Ciurdarescu *et al.*, 1998 and Chen *et al.*, 1999**). The present study showed that the highest similarity (82.4%) was observed between *T. harzianum* (FE_2) and *T. harzianum* (FE_4), while the lowest (29.6%) was obtained between *T. harzianum* (FE_1) and *T. viride* (FE_6). *Trichoderma harzianum* (FE_4) was more close to *T. harzianum* (FE_2) (the similarity 82.4%) also, *T. harzianum* (FE_3) was more close to *T. harzianum* (FE_1) at the similarity of 77.8%. While, *T. Koningii* (FE_5), *T. viride* (FE_6), reference strain *T. koningii* (I_7) or reference strain *T. harzianum* (I_8) clustered alone.

IV- Randomly Amplified Polymorphic DNA (RAPD)

A total of 99 DNA bands (7 monomorphic and 92 polymorphic) were detected for the eight *Tricoderma* isolates and generated by the 9 random primers (Table 7) and (Figure 5). Few bands were common (monomorphic for all isolates), three bands for primer OPA06 and four bands for primer OPA07. The use of different primers revealed different levels of polymorphism. The number of amplified DNA fragments was scored for each primer. Primer OPA19 amplified the highest number of amplicons (18), all were polymorphic among the eight *Trichoderma* isolates, while the lowest number (5) was amplified when the primer OPA06 was used. The number of polymorphic amplicons per primer ranged from 2 (primer OPA06) to 18 (primer OPA19) with an average of 10 per primer.

The distance matrix (NJTREE) and the phenogram (TDRAW) among the eight *Tricoderma* isolates utilizing RAPD-PCR markers (Table 8 and Figure 6, respectively) were detected by RAPDistance package version 1.4 according to Dice (**Nei and Li, 1972**) matrix. The analysis was based on the number of bands that were different between any given pair of species. The strongest relationship was scored between *Trichoderma* isolates, FE_5 and FE_6 (similarity index 69%), while *Trichoderma* isolates, FE_3 and I_7 were the most genetically distant isolate (similarity index 32%). The phenogram tree showed that the *Trichoderma* isolates, FE_1, FE_2, FE_3 and FE_4 morphologically characterized as *Trichoderma harzianum* appeared in one cluster with the reference isolate I_8.The second cluster includes *Trichoderma* isolates, FE_5, FE_6 and I_7.

Table (7): Total number of amplicons, monomorphic amplicons, polymorphic amplicons and percentage of polymorphism a revealed by RAPD markers among the eight *Trichoderma* isolates.

Primer	Total # of amplicons	Monomorphic amplicons	Polymorphic amplicons	% of polymorphism
OPA02	10	0	10	100
OPA03	13	0	13	100
OPA04	13	0	13	100
OPA06	5	3	2	40
OPA07	7	4	3	43
OPA16	10	0	10	100
OPA18	13	0	13	100
OPA19	18	0	18	100
OPA20	10	0	10	100
Total	99	7	92	
Average	11	0.78	10	87

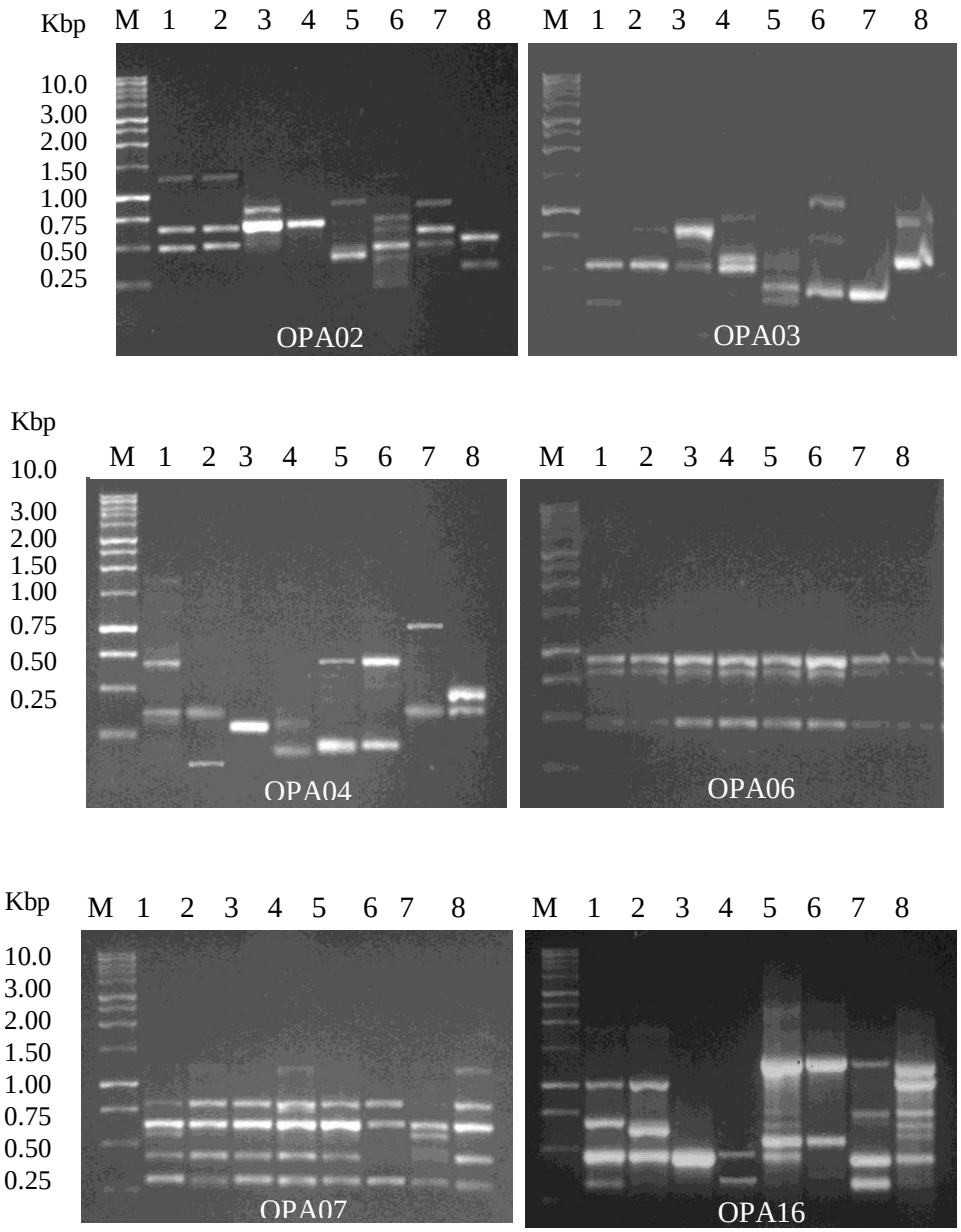


Fig. (5a): RAPD–RCR Patterns of eight *Trichoderma* isolates using primers, OPA02, OPA03, OPA04, OPA06, OPA07 and OPA16. Lane 1-8 represent isolates FE_1, FE_2, FE_3, FE_4, FE_5 FE_6, I_7 and I_8, respectively. Lane M represents the molecular size marker (1 kb leader).

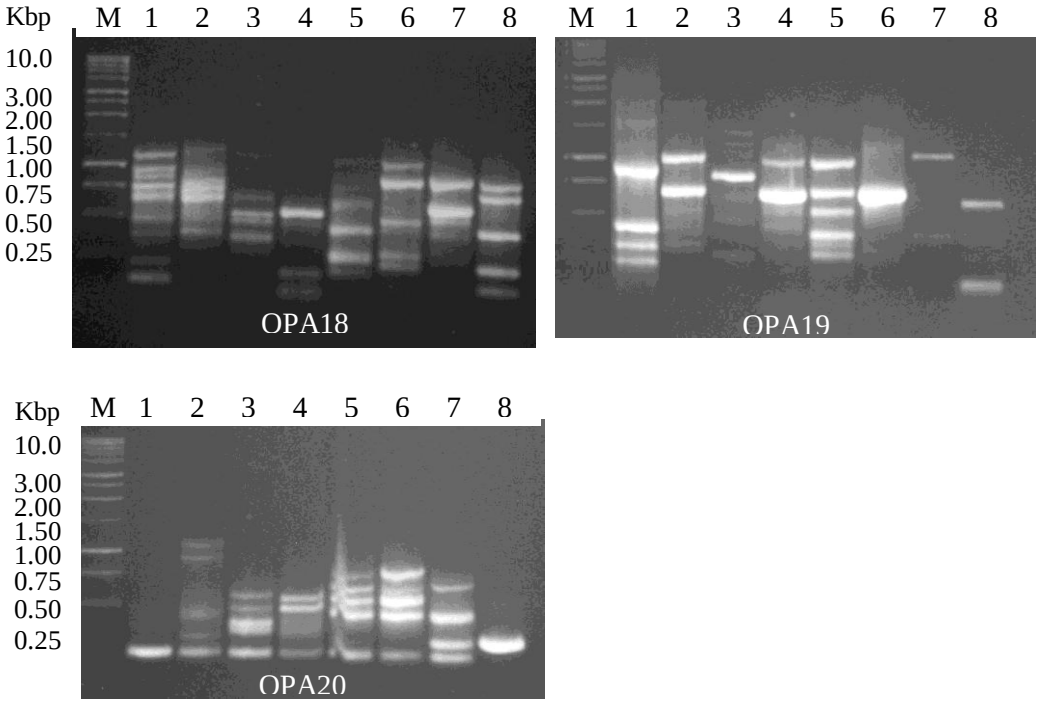


Fig. (5b): RAPD–RCR Patterns of eight *Trichoderma* isolates using primers, OPA18, OPA19 and OPA20. Lane 1-8 represent isolates FE_1, FE_2, FE_3, FE_4, FE_5 FE_6, I_7 and I_8, respectively. Lane M represents the molecular size marker (1 kb leader).

Table (8): Similarity indices calculated by RAPDistance package among *Trichoderma* isolates.

***Trichoderma* isolates**	**FE_1**	**FE_2**	**FE_3**	**FE_4**	**FE_5**	**FE_6**	**I_7**	**I_8**
FE_1	**100**							
FE_2	66	**100**						
FE_3	49	50	**100**					
FE_4	46	50	54	**100**				
FE_5	53	52	50	52	**100**			
FE_6	39	39	39	41	69	**100**		
I_7	43	49	32	43	49	52	**100**	
I_8	43	55	41	49	51	33	52	**100**

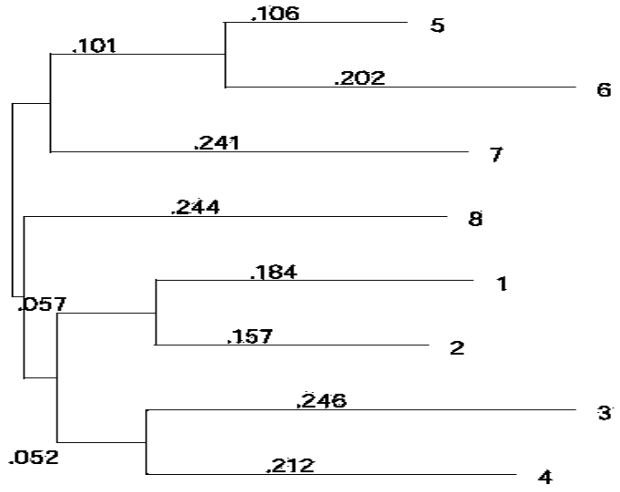


Fig. (6): Phenogram demonstrating the relationships among eight *Trichoderma* isolates based on a compiled data set.

The RAPD–PCR analysis showed a high level of sequence similarity between the eight *Trichoderma* isolates initially tested, indicating a low level of genetic heterogeneity between *Trichoderma* isolates. This technique has already been employed effectively for assessing the degree of genetic variation in a range of *Trichoderma* fungi (**Kuhls *et al*., 1999; Anjaiah *et al*., 2001, Goes *et al*., 2002 and Latha *et al*., 2002**).

Until recently, *Trichoderma* spp. were being identified on bases of morphological data only. However, subsequent molecular analysis of several strains including some ex-type strains revealed that classification based on morphological data have been, to a great extent, erroneous, resulting in reclassification of several isolates and species using molecular tools. (**Kuhls *et al*., 1997; Bulat *et al*., 1998; Castle *et al*., 1998; Lieckfeldt *et al*., 1999; Hermosa *et al*., 2000 and Latha *et al*., 2002**). The present study showed that the highest similarity (69%) was observed between *T. viride* (FE_6) and *T. Koningii* (FE_5), followed by 66% between *T. harzianum* (FE_2) and *T. harzianum* (FE_1) and 54% between *T. harzianum* (FE_4) and *T. harzianum* (FE_3). *Trichodrma harzianum* (FE_4) is more close to *T. harzianum* (FE_3), also *T. harzianum* (FE_2) was closer more to *T. harzianum* (FE_1) and *T. Koningii* (FE_5) was closer more to *T. viride* (FE_6). The reference strain *T. harzianum* (I_8) clustered with *T. harzianum* and the reference strain *T. koningii* (I_7) clustered with *T. Koningii* and *T. viride*.

Identification of the *Trichoderma* Isolates by Unique Biochemical and Molecular Markers

Unique markers obtained by different markers (protein and RAPD) were used in the present study to characterize the eight *Trichoderma* isolates. Unique markers are defined as bands that specifically identify isolate from the others by their presence or absence. As shown in Table (9), the total specific markers generated by biochemical analysis were 5. A number of 4 bands were scored as negative markers, while one was scored as positive marker. The eight *Trichoderma* isolates were characterized by 35 positive and 2 negative unique RAPD markers.

Table (9): isolate identification by unique biochemical marker and unique RAPD markers among each of the eight *Trichoderma* isolates.

Isolate	Biochemical markers			RAPD markers		
	Positive	Negative	Total	Positive	Negative	Total
FE_1				OPA03-300 bp OPA04-1750 bp OPA04-1500 bp OPA04-1200 bp OPA19-2000 bp OPA19-650 bp		6
FE_2				OPA03-1400 bp OPA19-1900 bp OPA19-1600 bp OPA20-1100 bp OPA20-900 bp		5
FE_3				OPA02-850 bp OPA03-<250 bp OPA19-1400 bp OPA19-850 bp OPA19-250 bp		5
FE_4				OPA03-<250 bp OPA03-<250 bp OPA06-350 bp OPA18-<250 bp OPA19-<250 bp		5
FE_5	50.46 kDa		1	OPA03-350 bp OPA03-250 bp OPA16-2100 bp		3
FE_6		32.91 kDa 13.91 kDa	2	OPA02-250 bp OPA03-1200 bp OPA16-350 bp OPA18-300 bp OPA20-800 bp		5
I_7		14.94 kDa	1	OPA04-900 bp OPA07-500 bp OPA19-1100 bp		3
I_8		224.0 kDa	1	OPA02-600 bp OPA02-375 bp OPA19-<250 bp	OPA06-600 bp OPA20-250 bp	5
Total	1	4	5	35	2	37

The least number of RAPD-PCR markers was detected for primers OPA06 and OPA07 (one marker out of 5 and 7 amplified bands, respectively), while the largest number of RAPD-PCR markers was detected for primer OPA19 (10 markers out of 18 bands)

Morphological analysis is highly prone to error, and consequently roughly 50% of the *Trichoderma* spp. deposited in culture collections under names obtained by morphological analysis alone are wrong. As a solution to this problem, they have recently developed a DNA–barcode system for quick

identification on the basis of defined nucleotide sequence differences in the ITS_1 and ITS_2 region (**Druzhinina *et al*., 2004**).

In conclusion, the RAPD-PCR analysis used in the present study could successfully characterize the eight *Trichoderma* isolates and determine a specific molecular markers.

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التوصيف البيوكيميائي والجزيئى لبعض عزلات من فطر الترايكودرما المضاد للفطر رايزوكتونيا سولانى المسبب لعفن جذور الفاصوليا

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يستخدم جنس الترايكوديرما كمواد بيولوجية فعالة ضد العديد من فطريات التربة الممرضة للنبات، ومن الصعب تمييز العديد من العزلات ذات التحكم البيولوجى العالى عن غيرها من العزلات. ولهذا تم استخدام طريقتين من الواسمات: البروتين وال RAPD لدراسة التباينات الوراثية بين ست عزلات من فطر الترايكوديرما والتى سبق عزلها من التربة الملامسة لجذور نباتات مختلفة منزرعة بمحافظة الفيوم وسلالتين مرجعيتين هما *Trichoderma harzianum* ، *T. koningii* تم الحصول عليها من كلية الزراعة جامعة عين شمس.

أظهر التوصيف المورفولوجى وجود أربع عزلات *T. harzianum* ، عزلة واحدة من *T. koningii* و أخرى من *T. viride* . وبدراسة كفاءة فطر الترايكوديرما فى التضاد لفطر الرايزوكتونيا سولانى اتضح أن أفضل العزلات كانت تتبع *T. harzianum* . أمكن التحكم فى مرض ذبول بادرات نبات الفاصوليا بإضافة عزلات فطر الترايكوديرما وكذلك راشح مزارعها الى تربة سبق تلقيحها بمزارع فطر رايزوكتونيا سولانى.

أظهرت نتائج التحليل الوراثى البيوكيماوى والجزيئى نسبة تباين قدرها ٩٦,٨% و٩٢,٩٣% لكل من البروتين وال RAPD على التوالى. تم تقدير العلاقات الوراثية بين عزلات الترايكوديرما الثمانية باستخدام معامل Dice، ولقد تراوحت نسبة التشابه الوراثى مابين ٢٩,٦% الى ٧٧,٨% و٣٢% الى ٦٩% للبروتين والـ RAPD على التوالى. كما أوضحت نتائج تحليلات درجات القرابة الوراثية للبروتين أن العزلة *T. viride* (FE_6) تقع فى مجموعة منفردة عن باقى العزلات السبع الاخرى. واتضح من درجات القرابة الوراثية لـل RAPD أن جميع عزلات *T. harzianum* (FE_1,FE_2, FE_3, FE_4, I_8) تقع فى مجموعة منفردة عن باقى العزلات الاخرى.

وأظهرت البيانات الناتجة عن تقانة التحليل الوراثى البيوكيماوى للبروتين ٥ واسمات فريدة ميزت ٤ عزلات من الثمانية عزلات التى شملتها الدراسة بينما أظهرت بادئات ال RAPD قدرة على تمييز جميع العزلات الثمانية باستخدام ٩ بادئات عشوائية.

وقد استنتج من هذه الدراسة إمكانية نجاح استخدام تكنيك ال RAPD فى توصيف عزلات فطر الترايكوديرما وتحديد واسمات جزيئية خاصة بها.